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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JAN 31 1975

Application for Research Grant
(Use extra pages as needed)

Date: 1-20-75

1. Principal Investigator (give title and degrees):

Theodore Alan Slotkin, Ph.D.
Assistant Professor of Pharmacology

2. Institution & address:

Duke University Medical Center
Department of Physiology and Pharmacology
Durham, North Carolina 27710

3. Department(s) where research will be done or collaboration provided:

Department of Physiology and Pharmacology

4. Short title of study:

EFFECTS OF NICOTINE ON SYMPATHO-ADRENAL DEVELOPMENT

5. Proposed starting date: 7/1/75

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

Many autonomic drugs and drugs which act in the CNS exert similar direct or reflex effects on catecholamine stores in adrenergic neurons and in the adrenal medulla of the mature organism; however, the actions of these drugs are often markedly different (and even opposite) in fetal or neonatal animals. The proposed studies will determine how maturational changes in the rat adrenal medulla alter the effects of nicotine on that tissue. In addition, since the maturation of the adrenal medulla is itself dependent upon biochemical and neuronal feedback mechanisms, this study will also examine how pre- and post-natal exposure to nicotine alters the subsequent maturation of normal sympatho-adrenal function.

In the time period outlined, we expect to determine:

(a) The acute and chronic effects of nicotine on the mature adrenal medulla (already in progress).

(b) The effects of acute and chronic nicotine on post-natal adrenergic development.

(c) The long-term effects of pre-natal exposure to nicotine on the developing adrenal medulla.

(d) Whether pretreatment with nicotine blocking agents alters developmental effects of nicotine.

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8. Brief statement of working hypothesis:

The adrenergic nervous system and adrenal medulla play vital roles in the maintenance of the cardiovascular system, normal metabolic function and in the response to various stress situations. In the neonate, these tissues are markedly immature in their capability to synthesize, store and secrete catecholamines. The deficiencies in the adrenal medulla result in part from age-dependent alterations in the function and contents of storage vesicles, which are in turn dependent upon neural stimulation. These studies will elucidate many of the factors operating in the development of sympathetic amine stores. The rates of synthesis of adrenal catecholamines, storage vesicles, and the development of storage capabilities will be measured as well as the functioning of the adrenal gland in response to neural stimulation and nicotine-induced amine depletion. Data from several studies suggest that there are crucial differences between adults and neonates in amine storage capabilities and in the effects of drugs (such as nicotine) which act either directly on the adrenal or which elicit reflex stimulation of the sympatho-adrenal axis. Using the adrenal medulla as a model, some of these differences in drug action can be elucidated.

Fetuses are often exposed to a wide variety of drugs known to affect catecholamines, either as a result of clinical treatment or maternal self-

(continued on page 6)

9. Details of experimental design and procedures (append extra pages as necessary)

(a) Introduction

1. Previous work done by applicant:

The major effort of the applicant has been directed toward elucidating the mechanisms of regulation on the synthesis, uptake, storage and release of the catecholamines of the adrenal medulla. Besides the inherent interest in studying this tissue, the adrenal medulla is often utilized as a model of the adrenergic neuron; both tissues arise embryonically from the neural crest and both have the ability to synthesize, store and secrete catecholamines. Each contains storage vesicles which can accumulate amines by a mechanism which is stimulated by ATP-Mg^{2+} and blocked by reserpine. The vesicles contain dopamine β-hydroxylase (DBH), chromogranins and adenine nucleotides as well as catecholamines; it is accepted generally that the catecholamine and adenine nucleotides (primarily ATP) form a storage complex in a molar ratio of 4 to 1.

Techniques developed to a large extent in this laboratory enable the evaluation of many of the factors which influence catecholamine disposition from the point of view of alterations in storage vesicles. Initial studies concentrated on the mechanism by which storage vesicles are assembled administration of drugs (insulin, reserpine) which elicit reflex sympatho-adrenal discharge led to the formation of large numbers of new, "immature" vesicles which had properties different from the normal population (Slotkin and Kirshner, 1973a,b; Slotkin and Edwards, 1973):

(1) The new vesicles showed a decreased preference for uptake of epinephrine vs. metaraminol.
(2) There was a decreased buoyant density in the new vesicles.
(3) There was a decreased ratio of catecholamines to ATP in the new vesicles.
(4) The catecholamine content per vesicle was decreased.

Another situation in which large numbers of new vesicles have to be synthesized is in maturation. Studies from other laboratories (Ignarro and Shideman, 1968; Waymire et al., 1974; Daikoku et al., 1969; Elfvin, 1967) indicated that in rats and chicks, catecholamine biosynthetic enzymes appeared early in gestation but catecholamines were not detectable until much later, when storage vesicles could first be detected by electron microscopy (18th day of gestation). Consequently, most of the maturational increases in catecholamines occur postnatally.

(Continued on page 6).

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Laboratory consists of 1300 square feet fitted with standard laboratory type benches. Major items of equipment include: Sorvall RC-2B centrifuge, Beckman L5-50 ultracentrifuge and rotors, Farrand ratio fluorometer, catecholamine autoanalyzer, Wang 600-6-TP programmable calculator, incubation baths, pH meter, balances and other general items of laboratory hardware. Research facilities include animal rooms, cold rooms, a spectrophotometer and a liquid scintillation counter.

11. Additional facilities required:

The current RC-2B is in heavy, continuous use by five people in this laboratory and is quite old. Funds are therefore requested for another centrifuge for the proposed studies.

12. Biographical sketches of investigator(s) and other professional personnel (append):

See pages 17, 18, 19 and 20.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See page 21.

4.

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Theodore A. Slotkin - Principal Investigator	50	0
Technical		
Christopher Lau (B.S.)	80	REDACTED
Fringe Benefits (12.6%)		
Sub-Total for A		R

B. Consumable supplies (by major categories)

Animals, including housing and shipping costs (100 male rats @ $3.00, 100 pregnant rats @ $10.00, $200 shipping, housing @ 10¢/day for avg. 50 days)	2500
Radioisotopes (^{3}H-epinephrine, metaraminol, tyramine; ^{14}C-tyrosine & epinephrine)	1750
Chemicals and hardware	750
Sub-Total (B)	5000

C. Other expenses (itemize)

Equipment maintenance and service	500
Travel to ASPET Spring and Fall Meetings	500
Sub-Total for C	1000
Running Total of A + B + C	R

D. Permanent equipment (itemize)

Sorvall RC-2B centrifuge and rotors	4000
Sub-Total for D	4000
E	1873
Total request	$18359

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	5000	1000	0	1931	14807
Year 3		5000	1000	0	1994	15282

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
1. Effects of morphine on the adrenal medulla	DA-00465	$85,000	6/1/73-5/31/76
2. Hypertensive rats	American Heart Assn.	$50,000	10/1/73-9/30/76

These projects are unrelated to the present proposal and provide salary support for the investigator and two post-doctoral fellows and laboratory expenses for these projects.

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Theodore Alan Slotkin

Signature Theodore Slotkin Date 1/20/75

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Area Code Number Extension

Checks payable to

Duke University

Mailing address for checks

Vice President, Business & Finance

Duke University

Durham, North Carolina 27706

Responsible officer of institution:

Typed Name W. G. Anlyan, M.D.

Title Vice President for Health Affairs

Signature W G Anlyan Date 1/28/75

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8. (continued from page 2)

administration; although catecholamine stores undergo marked maturational changes, little is known about the effects of prenatal drug exposure on the subsequent developmental process. A number of studies suggest that even single doses of drugs at critical stages in development may alter permanently the disposition of catecholamines in central and peripheral adrenergic tissue. The proposed experiments will identify on the biochemical and functional levels, specific loci of nicotine-induced maturational alterations, and will show whether pretreatment with nicotinic blocking agents can prevent the long-term effects of nicotine. These studies may be of considerable importance in evaluating the consequences of exposure to nicotine during pre- and postnatal development.

9. (continued from page 2)

(a) Introduction

1. Previous work done by applicant (con't.):

Recent studies by the applicant (Slotkin, 1973a,b; 1974a) examined the maturation of the rat adrenal medulla from the point of view of changes in the number and properties of storage vesicles. This first entailed modification of the available methodology to enable measurements utilizing small amounts of tissue; the methods are described in detail in the appended reprints and in the "Methods" section.

At birth, the number of storage vesicles (as determined by DBH, an enzyme marker for vesicles) was quite low, and the vesicles differed markedly from adult vesicles in that they were considerably more dense on sucrose density gradients. The density difference was confirmed by determination of the subcellular distribution of catecholamines - lower levels were found in fractions usually containing "light" vesicles. Other properties of the vesicles were found to be different from adults neonatal vesicles were more fragile and displayed greater susceptibility to osmotic shock; however, the stability of amine storage was normal as was the ratio of catecholamines to ATP (the putative components of the storage complex). On the functional level, the uptake of amines into the vesicles appeared to be normal, but neonates given insulin did not evidence a normal catecholamine secretory response; however, neonatal adrenals excised and incubated in high-potassium Locke's solution did secrete normally, indicating that the lack of secretion in vivo is probably due to non-functional innervation of the gland by the splanchnic nerve.

Sometime between birth and 10 days of age, a complete alteration of sympatho-adrenal function occurs: the secretory response becomes normal, "light" vesicles appear, DBH, ATP and catecholamines increase markedly and the vesicle density shifts such that vesicles are now lighter than in adults. Furthermore, at this stage there is a partial loss of normal preference for uptake of catecholamines vs. non-catecholamines (metaraminol) and the stability of storage is impaired. These are all signs of increased neural input to the gland and the vesicles are similar to those found in stressed adult animals (in which increased neural stimulation also occurs). These qualitative and quantitative alterations in function and properties gradually approach those in normal adults by 40-50 days of age, after which additional altera-

tions reflect only increases in the number of vesicles.

To summarize these results, the maturation of the adrenal medulla is dependent upon neural input to the gland. At birth, no input occurs, vesicle turnover is low, and dense, overloaded vesicles are present. By 10 days, innervation is functional, turnover increases markedly, and "light" vesicles appear with altered uptake and storage capabilities.

Additional studies have been conducted to examine these changes in terms of kinetic analysis of uptake. The Km for epinephrine (E) remained at about 30 μM throughout development but the maximal uptake (Umax) at birth was 30 nmols/30 min/100 μg endogenous catecholamines compared to 20 nmols at subsequent ages. The Km for metaraminol (MA) was about 4 mM at birth, 0.7 mM at 10-20 days of age and 1.2 mM thereafter, while Umax was approximately 170 nmols initially, 50 nmols at 10-20 days and 70 nmols thereafter. The preference of the vesicles for uptake of E vs. MA (both at 0.1 mM) was approximately 6 to 1 at birth, 1 to 1 at 10 days, 3.5 to 1 at 20 days and 5 to 1 thereafter, while the preference for E vs. tyramine was 2 to 1 except at 10 days, when the ratio was 1 to 1. These data suggest that the two vesicular uptake systems, typified by incorporations of E and of MA, develop independently of each other and that the age-dependent alterations are present both at the level of the vesicle membrane and intravesicular storage. A kinetic model was developed to aid in the identification of probable sites of specific age-dependent alterations. Maturational changes in uptake and specificity produced parallel changes in synthesis of catecholamines as determined by conversion of newly-incorporated tyramine to octopamine. Furthermore, these alterations could affect the actions of adrenergic false transmitters (which displace catecholamines from vesicles) on immature rats.

These data indicate that any study of drug effects on development of catecholamine stores (such as this proposal) must comprise at least three elements:

(1) Evaluation of the effect of the drug in adults;
(2) Evaluation of how the maturational alterations change the action of the drug in the developing organism; and
(3) Evaluation of how drug administration alters subsequent development of catecholamine stores.

One drug which we are presently studying in this fashion is morphine (Anderson and Slotkin, 1974; Slotkin and Anderson, 1974a,b). Morphine was administered twice daily to adult rats and the adrenals were analyzed for catecholamines (CA), tyrosine hydroxylase (TH) activity and dopamine β-hydroxylase (DBH) activity. Twenty-four hours after dosage with 10 mg/kg, CA were reduced and TH and DBH slightly elevated. After one week of treatment, all 3 were elevated. Subsequent dose increments resulted in acute decreases in CA and DBH and increases in TH, followed by dose-dependent increases in CA, DBH and TH to a maximum of 2-3 times control levels after 2 weeks at 100 mg/kg. Withdrawal led to a decline to control levels within 10 days. Shifts in the subcellular distribution of DBH suggested that morphine administration increased the rate of vesicle synthesis, and measurements of the uptakes of ^{14}C-epinephrine and ^{3}H-metaraminol into isolated storage vesicles indicated an increased proportion of "immature" vesicles. Prolonged administration of large doses of morphine led to the formation of vesicles with an apparently defective amine uptake mechanism; these vesicles also displayed abnormally low fragility and a reduced rate of spontaneous CA efflux. These data suggest that (1) tolerance to morphine-induced sympatho-adrenal discharge does not develop and that the recovery from

the acute CA depletion results from increased CA synthesis and storage, and (2) morphine produces a persistent change in the properties of adrenal medullary storage vesicles.

On the other hand, preliminary studies with perinatally-addicted developing rats demonstrated changes completely different from those seen in adults; catecholamine levels and dopamine β-hydroxylase activity were reduced compared to controls and no induction of tyrosine hydroxylase was observed. The time course of adrenomedullary maturation was delayed through the first 10-20 days of age, with reduced numbers of storage vesicles and larger proportions of partially filled vesicles. On exposure to morphine, continued until weaning, perinatally addicted rats did not display any of the changes in catecholamine synthesis or uptake seen in adult rats, but we have preliminary evidence that defects may develop post-weaning even if morphine is discontinued. The differences between adults and developing rats can be partly explained by the absence of functional innervation of the neonatal adrenal medulla, but the inability of morphine to cause induction after 10 days may indicate a "re-programming" of the relationship between neural input and development of the adrenal medulla.

Additional studies are being conducted with adult and developing spontaneously hypertensive rats (SHR), where a genetic abnormality (hypertension) is associated with alterations in sympatho-adrenal catecholamine disposition (Slotkin and Green, 1974; Slotkin, 1974b). Specific loci of alterations in synthesis, uptake, storage and release have been identified in a fashion analogous to studies with drugs.

Current research efforts are concentrating on the elucidation of the acute and chronic effects of nicotine on the mature rat adrenal. Preliminary results indicate a similarity to the effects of other sympatho-adrenal stimulators, such as morphine, reserpine and insulin; however, tolerance appears to develop upon prolonged exposure (several weeks). Tyrosine hydroxylase, dopamine β-hydroxylase and catecholamine levels are all elevated during chronic exposure, and storage vesicles with a defective uptake system are detectable.

2. Previous work by others:

A vast literature exists on the effects of drugs on central and peripheral catecholamine disposition, and this discussion will be limited to those concerning the development of the adrenal medulla and the actions of drugs on developing adrenergic tissue.

During prenatal and postnatal development, there is a marked increase in catecholamine levels in adrenergic neurons, and in the adrenal medulla, as well as changes in the levels of catecholamine synthesizing enzymes (Ignarro and Shideman, 1968; Heggeness et al., 1970; Iversen et al., 1967; Hökfelt, 1951; Mirkin, 1972; Patrick and Kirshner, 1972). In chick embryos, tyrosine hydroxylase, the rate-limiting enzyme in catecholamine formation, is present on the first day of gestation, and all the enzymes are present by the sixth day. Despite the presence of the necessary enzymes, adrenal catecholamines do not begin developing until several days before birth in the rat (gestational period = 22 days); electron microscopy of adrenal tissue reveals the presence of a small number of storage vesicles four days before birth, with the subsequent appearance (two days before birth) of catecholamines (Daikoku et al., 1969; Elfvin, 1967). These data

suggest that the presence of storage vesicles is a determining factor in the increase in adrenal catecholamines.

Because the storage vesicles appear relatively late in gestation, the largest changes in catecholamine content occur in the postnatal period. At this stage, neural input plays a role in determining the rate of development in amine stores. Thus, prior sectioning of the splanchnic nerve decreases the rate of development of adrenal catecholamines as well as the age-dependent increases in tyrosine hydroxylase (Patrick and Kirshner, 1972); in sympathetic ganglia, development of catecholamine biosynthetic enzymes is retarded by chronic administration of ganglionic blocking agents (Black, 1973). Neural input similarly accelerates the rate of recovery of amines in adult rats whose stores have been depleted by reserpine treatment (Patrick and Kirshner, 1971a,b). The post-natal dependence on neural input for development of amine stores is paralleled by the development of neurally-dependent response to stress. For example, secretion of catecholamines in response to asphyxia is largely independent of neurogenic stimulation in fetal calves, but is highly nerve-dependent two to three weeks after birth (Comline and Silver, 1966). This indicates that the development of catecholamines is accompanied by fundamental changes in the ability of the animal to utilize the amine stores.

Differences between adult and neonatal catecholamine stores are also apparent in their susceptibility to modification by drug treatment. Infant rats are more sensitive to the catecholamine depleting effects of reserpine and tetrabenazine (Kulkarni and Shideman, 1966). In addition, in chicks hatched from reserpine-treated eggs, following the initial depletion there is a long-lasting (weeks) increase in brain catecholamines, suggesting that alteration of catecholamine levels during development produces a prolonged alteration of catecholamine synthesis and/or storage systems (Sparber and Shideman, 1970). Additionally, drugs which do not ordinarily cross the blood-brain barrier in adults are often able to do so in fetal or neonatal animals (Liuzzi et al., 1974) with the result that peripherally active agents (such as guanethidine or 6-hydroxydopamine) administered systematically produce marked effects in the developing brain which are totally different from the lack of activity in mature brain. Surprisingly, almost no work has been done on the actions of nicotine on adrenergic development on the biochemical level.

The developmental aspects of catecholamines in central and peripheral adrenergic neurons appear to be quite similar to those of the adrenal medulla (Coyle and Axelrod, 1971, 1972a,b; Sachs, 1970), with the largest increases in catecholamines, enzymes and synaptosomal uptake mechanisms occurring in the first few weeks after birth in rodents and chicks. The maturational increases in catecholamines appear to follow the appearance of soluble vesicular proteins (Mirkin, 1974), indicating that the vesicles may also play a determining role in neuronal development. However, most studies of drug effects on adrenergic development have concentrated only on measuring amine levels, and in some cases, tyrosine hydroxylase and dopa decarboxylase activities. Thus, while a number of drugs (nicotine, chlorpromazine, iproniazid, reserpine, morphine, amphetamine, catecholamines, etc.) have been shown to produce long-lasting or permanent alterations in norepinephrine levels in various brain parts, in most of these cases experimental evidence for a specific biochemical lesion has not been

obtained, nor have any experiments been conducted to determine whether there are functional alterations in catecholamine disposition (synthesis, uptake, storage, release) (Vernadakis, 1974; Liuzzi et al., 1974). For instance, prenatal administration of chlorpromazine to rats shortly before birth leads ultimately to an increased brain norepinephrine level in adulthood (Nair, 1974), but the increases could represent increased synthesis, a greater storage capacity, enhanced uptake, reduced degradation, or reduced release. Administration of guanethidine to newborn mice results in long-lasting reductions of brain catecholamines, but again the mechanism or functional consequences of these developmental changes have not been determined (Liuzzi et al., 1974). Furthermore, no studies have been conducted to see whether the effects of drugs on adrenergic development can be prevented by pretreatment with blocking agents. Obviously, a detailed biochemical profile of enzymes, storage vesicles, uptake and release is required to elucidate the effects of drugs on adrenergic maturation.

(b) Methods

Drug dosage, adult rats.

Sprague-Dawley rats (Zivic-Miller) will be injected subcutaneously twice daily with aqueous solutions of nicotine (1 or 10 mg/kg) or nicotine in oil (2 or 20 mg/kg once daily). In some cases, to differentiate direct vs. reflex effects, rats in acute studies will be pretreated with chlorisondamine (10 mg/kg, s.c.), which blocks neural stimulation of the adrenal medulla; for chronic studies, reflex effects will be prevented by prior sectioning of the left splanchnic nerve while the contralateral adrenal remains innervated to serve for comparison of direct (denervated) vs. direct + reflex (innervated) effects. Animals will be sacrificed at intervals of several hours to several weeks, as well as in the post-exposure period after discontinuing drug administration.

Studies also will be carried out in vitro to determine whether there is any direct effect on catecholamine biosynthetic or degradative enzymes (tyrosine hydroxylase, dopa decarboxylase, dopamine β-hydroxylase, monoamine oxidase) or whether they affect uptake or storage of catecholamines in isolated adrenal medullary vesicles. These studies will identify the direct and indirect biochemical effects of nicotine in mature animals and will thus serve as a model with which to compare drug activities in developing rats.

Drug dosage, developing rats.

In general, the dosages administered to pregnant rats (to expose the offspring in utero) may have to be somewhat lower than in non-pregnant adults, since the LD_{50} of many agents appears to be reduced. In other experiments, normal or prenatally exposed neonates will be given nicotine acutely or chronically either by direct administration or via the milk during chronic administration of the drug to the mother. In the latter experiments, additionally we will differentiate between purely prenatal and purely postanatal effects of nicotine by switching at birth prenatally-exposed pups to normal mothers and normal pups to mothers who will continue to receive nicotine.

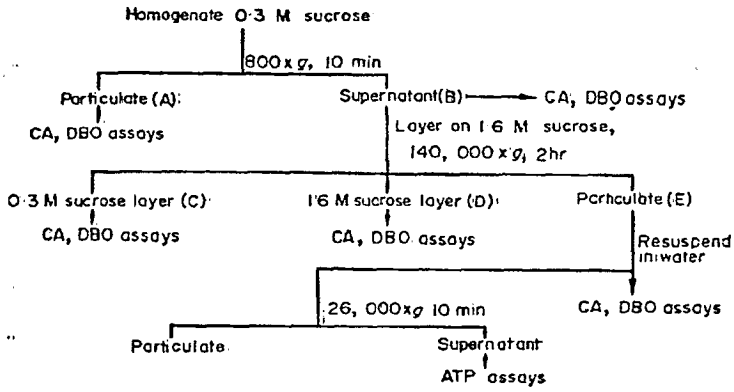
Offspring will be sacrificed at intervals of several days from

birth to young adulthood (40-50 days). These experiments will determine whether the effects of nicotine are different in developing rats compared to adults and will also indicate whether nicotine affects the biochemical development of catecholamine stores and whether permanent changes in catecholamine disposition can be produced by exposure to nicotine early in development.

Data collection

(a) Determination of the number and contents of storage vesicles:

The adrenal glands from each animal are excised, cleaned of fat and connective tissue, and homogenized (glass-to-glass) in 2.5 ml of ice-cold sucrose-Tris [300 mM sucrose containing 25 mM Tris (pH 7) and 0.1 mM iproniazid (to irreversibly inhibit monoamine oxidase)]. The suspension is centrifuged at 800 g for 10 min and the supernatant is decanted. The pellet is resuspended by glass-to-glass homogenization in 5 ml of distilled water and analyzed for catecholamines and dopamine β-hydroxylase (fraction A). One ml of supernatant is added to 1 ml water, homogenized and assayed (fraction B). Another ml of the 800 g supernatant is layered over 2.5 ml of 1.6 M sucrose containing 500 units/ml of beef catalase (Sigma) and centrifuged for 2 hr at 140,000 g in the No. 40 rotor of the Beckman model L5 ultracentrifuge. This separates intact heavy vesicles from light broken vesicle membranes and from most mitochondrial and lysosomal contaminants (Smith and Winkler, 1967). The 0.3 M sucrose layer (fraction C) and the 1.6 M sucrose layer (fraction D) are diluted with water to final volumes of 2 and 4 ml, respectively, homogenized, and assayed for catecholamines (CA) and dopamine β-hydroxylase (DBH). The vesicular pellet (fraction E) is resuspended in 2 ml water and homogenized to lyse the vesicles. One ml is removed for the determination of CA and DBH, and the remainder is centrifuged at 26,000 g to remove the vesicle membranes; the supernatant of this latter centrifugation is analyzed for ATP. A flow sheet of this procedure appears below.



DBH is an enzyme associated only with storage vesicles (Viveros et al., 1969a), and the determination of the distribution of DBH activity therefore provides a measure of the number of storage vesicles present as intact heavy vesicles, light vesicles and broken vesicle membranes. The low levels of DBH present early in development require the pooling of glands from several animals to obtain sufficient enzyme activity.

The ratio of catecholamines/DBH provides a measure of the sequence of synthesis of vesicles: new vesicles are deficient in catecholamines and therefore exhibit low ratios. This phenomenon is observed in adult rats in which adrenal stimulation is evoked (Slotkin and Kirshner, 1973a,b) and in developing rats during the stage immediately following the appearance of functional splanchnic innervation (Slotkin, 1973a,b); these data thus provide a sensitive measure for detecting drug-induced alterations in the number and rate of synthesis of vesicles.

ATP is an integral part of the catecholamine storage complex, and drug-induced alterations in ATP indicate changes in catecholamine binding ability. New vesicles and vesicles in developing rats display altered catecholamine to ATP ratios, and this is associated with changes in amine storage (Slotkin and Kirshner, 1973b); the ATP parameter thus provides another sensitive measure with which to compare drug effects in adults and developing rats.

The fraction of vesicles ruptured during homogenization is fairly constant from preparation to preparation (Slotkin and Kirshner, 1973a,b). The distribution of catecholamines and DBH in the broken vesicle fraction thus provide a measure of the mechanical fragility of the vesicles, which is dependent on properties of the vesicle membrane (Slotkin and Kirshner, 1973a,b; Anderson and Slotkin, 1974); the fragility has been observed to change after drug treatments and during development (Slotkin, 1973b; Anderson and Slotkin, 1974).

Alterations in any of the above factors -- catecholamines, DBH, number and distribution of vesicles, ATP levels, vesicle fragility -- can all be used as sensitive, functional indicators of altered catecholamine disposition.

(b) Determination of the uptake and storage capabilities of the vesicles:

One of the major functions of storage vesicles is the ability to incorporate catecholamines by an ATP-Mg^{2+}-stimulated uptake mechanism; non-catecholamines (metaraminol) are incorporated by a non-stimulated mechanism. The uptake mechanisms are known to be altered by nicotine and other drugs, and during the normal maturation process (Lundborg, 1966; Lundborg and Stitzel, 1967; Slotkin and Kirshner, 1973a,b; Slotkin and Edwards, 1973; Slotkin, 1973a; Anderson and Slotkin, 1974), and the evaluation of uptake and storage capabilities are therefore required in the proposed studies.

Adrenal glands from each animal are homogenized in 2.2 ml sucrose-Tris and an aliquot is withdrawn for catecholamine analysis. After centrifugation at 800 g for 10 min, the supernatant is decanted and 0.4 ml is pipetted into each of four tubes containing 0.1 ml of 50 mM ATP plus Mg^{2+}, 0.1 ml of 1 mM epinephrine and either 1 µCi ^{14}C-epinephrine or 5 µCi ^{3}H-metaraminol plus 0.1 ml of 1 mM metaraminol.

The added epinephrine is sufficient to eliminate any differences in extravesicular amine concentrations among the samples. Sucrose-Tris is added to each tube to give a total volume of 1 ml, and one epinephrine- and one metaraminol-containing sample are brought to 30° for 30 min; the duplicate tubes are kept at 0° for 30 min. Uptake is stopped by the addition of 2 ml of ice-cold sucrose-Tris, and the samples are centrifuged for 10 min at 26,000 g. The supernatants are decanted and added to an equal volume of 7% perchloric acid, centrifuged, and analyzed for CA and radioactivity. The vesicular pellet is washed with sucrose-Tris, centrifuged, rewashed, centrifuged, resuspended (glass-to-glass homogenization) in 3 ml of 3.5% perchloric acid, recentrifuged, and then analyzed for CA and radioactivity. Uptake in each sample is calculated as described below:

$$\text{Gross uptake per gland} = \frac{\text{counts per min in vesicles} \times \text{CA content per gland}}{\text{specific activity of labeling medium} \times \text{CA content of vesicles in sample}}$$

$$\text{Gross uptake per 100 µg CA} = \frac{\text{uptake per gland} \times 100}{\text{micrograms of CA per gland}}$$

The uptake at 0° is then subtracted from the uptake at 30° to give the temperature-dependent vesicular uptakes. Under these conditions, uptake occurs solely into storage vesicles (Slotkin and Kirshner, 1971, 1973b; Slotkin, 1973a).

Because "uptake" is a complex term (influx minus efflux), and since efflux is a measure of the stability of storage, only by the evaluation of efflux can an observed change in uptake be interpreted as altered influx or altered storage. To evaluate efflux, adrenals are homogenized in sucrose-Tris-iproniazid and centrifuged at 800 g. The vesicle-containing supernatant is labeled and washed twice as described above and resuspended in fresh sucrose-Tris. Sets of 15, 1-ml aliquots of labeled vesicles are brought to 37° to allow efflux to occur, and efflux is stopped by the addition of 2 ml of ice-cold sucrose-Tris after 0, 2.5, 5, 7.5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min. Samples are centrifuged for 10 min at 26,000 g and the supernatant solution is decanted and analyzed for CA and radioactivity. The vesicular pellets are resuspended in 3 ml of 3.5% perchloric acid (to lyse the vesicles), centrifuged 10 min at 26,000 g (to remove precipitated proteins), and analyzed for CA and radioactivity. The effluxes of endogenous CA, ^{14}C-epinephrine and ^{3}H-metaraminol are calculated as described previously (Slotkin et al., 1971).

These methods enable us to evaluate nicotine-induced changes in the development of the uptake and storage of amines in the vesicles.

It should be noted that the uptake of amines into isolated adrenal vesicles is not generally stereospecific (Carlsson et al., 1963). Radioactive dl-epinephrine is used rather than l-norepinephrine because epinephrine is the major catecholamine (80-90%) in the rat adrenal medulla.

(c) Buoyant densities of storage vesicles:

Catecholamines and nucleotides represent a significant fraction of the dry weight of the storage vesicles (Smith, 1968). Therefore,

it is not surprising that vesicles with altered contents display altered buoyant densities (Viveros et al., 1969a, 1971a,b; Slotkin and Kirshner, 1973a; Slotkin, 1973b; Anderson and Slotkin, 1974); vesicles with abnormal densities are commonly found in adult rats after administration of catecholamine-depleting agents (Slotkin and Kirshner, 1973b; Slotkin and Edwards, 1973; Anderson and Slotkin, 1974) and in maturation when large numbers of new vesicles appear (Slotkin, 1973b). Consequently, we will examine the effects of nicotine on the buoyant densities of vesicles from adult and maturing rats.

Continuous density gradients, hyperbolic from 1 to 2 M sucrose, are prepared with a modification (Slotkin and Kirshner, 1971) of the apparatus of Ayad et al. (1968). Sucrose (1 M) is pumped into a mixing chamber containing 2 M sucrose (15 ml/gradient) at a rate equal to 30 ml. Adrenal storage vesicles from control rats and from drug-treated rats are labeled with [^{14}C]epinephrine, [^{3}H]metaraminol, or [^{3}H]epinephrine, washed twice, and resuspended in sucrose-Tris. Mixtures of vesicles are prepared as follows: tube 1 contains [^{14}C]-epinephrine (control) + [^{3}H]metaraminol (control); tube 2, [^{14}C]-epinephrine (control) + [^{3}H]metaraminol (drug); and tube 3, [^{14}C]-epinephrine (control) + [^{3}H]epinephrine (drug). The mixtures (total volume, 1 ml/mixture) are then layered onto the gradients and centrifuged at 105,000 x g for 3 hr in a Spinco SW 25 rotor. This is sufficient time to permit equilibrium density to be reached. The gradient tubes are emptied dropwise from the bottom (20 drops/sample), and each sample is diluted with 2 ml of 5% perchloric acid, centrifuged at 26,000 g for 10 min, and analyzed for ^{14}C, ^{3}H, and catecholamines. This method enables detection of small differences in density because both control and drug-treated vesicles are spun in the same gradient.

(d) Development of functional innervation and nicotinic receptors:

Studies with developing rats indicate that functional innervation is lacking in neonates (Slotkin, 1973b). Since one signal for biochemical maturation is the level of neural input to the adrenal (Patrick and Kirshner, 1972; Slotkin, 1973b), it is important to establish whether a given drug accelerates or delays the onset of innervation. This can be tested by administration of 20 IU/kg of insulin s.c. which normally evokes reflex secretion of 30% of total catecholamines in 3 hr in innervated adrenals; if innervation is absent, no secretion occurs (Slotkin, 1973b). This effect is contrasted with the development of nicotinic receptors in the adrenal medulla, as determined by the ability to secrete in direct response to cholinergic drugs. These data will indicate whether nicotine alters the development of splanchnic innervation or of post-synaptic receptors.

(e) Analysis of catecholamine biosynthetic and degradative enzymes:

The development of the ability of the adrenal medulla to synthesize catecholamines generally follows the appearance of tyrosine hydroxylase and dopamine β-hydroxylase (Ignarro and Shideman, 1968; Patrick and Kirshner, 1972; Slotkin, 1973b; Slotkin and Anderson, 1974a). Glands from control or drug-treated adult or developing rats are homogenized in 2.5 ml of 0.15 M KCl and aliquots withdrawn for

determination of catecholamines, dopamine β-hydroxylase, dopa decarboxylase and monoamine oxidase. The remainder is spun at 26,000 g to sediment catecholamine storage vesicles to avoid interference with the tyrosine hydroxylase assay (26,000 g supernatant). This method enables the determination of catecholamines and all four enzymes from individual rats.

(f) Assays:

For catecholamines, 0.1 ml aliquots of all samples are added to 1.9 ml of 3.5% perchloric acid and centrifuged at 26,000 g x 10 min in order to remove precipitated protein. The supernatants are analyzed for catecholamines by the trihydroxyindole method, using an autoanalyzer (Merrills, 1963).

Radioactive amines are measured by liquid scintillation spectrometry. One milliliter of each sample is added to 10 ml of a 1:2 mixture of Triton X-100 detergent and toluene (containing 2,5-diphenyloxazole and p-bis[2-(5-phenyloxazolyl)]benzene).

Monoamine oxidase activity is measured by the procedure of Laduron and Belpaire (1968), using [^{3}H]tyramine (10 μM) as substrate. Dopamine β-hydroxylase is assayed by the method of Friedman and Kaufman (1965), using [^{3}H]tyramine (10 μM) as substrate. Tyrosine hydroxylase and dopa decarboxylase activity are measured by the method of Waymire et al. (1971), using [^{14}C]tyrosine (100 μM) or [^{14}C] dopa (33 μM) as substrate.

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13. Most recent and pertinent articles:

1. Anderson, T.R. and Slotkin, T.A. Maturation of the adrenal medulla. IV. Effects of morphine. Biochem. Pharmacol., in press.

2. Slotkin, T.A. Maturation of the adrenal medulla. III. Practical and theoretical considerations of age-dependent alterations in kinetics of incorporation of catechol- and non-catecholamines. Biochem. Pharmacol., in press.

3. Slotkin, T.A. Maturation of the adrenal medulla. II. Content and properties of catecholamine storage vesicles of the rat. Biochem. Pharmacol. 22:2033 (1973).

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5. Slotkin, T.A. Effects of reserpine on the adrenal medulla of the spontaneously hypertensive rat. Brit. J. Pharmacol., in press.

6. Slotkin, T.A. and Green, H.O. Adrenal medullary storage vesicles of the spontaneously hypertensive rat. Biochem. Pharmacol., in press.

Progress Report #4
July 1, 1974 - December 31, 1974

CTR Grant #864
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MATURATION OF THE ADRENAL MEDULLA: CATECHOLAMINE STORES IN NORMAL AND HYPERTENSIVE RATS

Earlier progress reports from this project detailed the biochemical maturation of the adrenal medulla in normotensive Wistar rats (NWR) as well as evaluating adrenal catecholamine synthesis, storage and release in adult spontaneously hypertensive rats (SHR), and therefore these results will not be discussed here. As described in progress report number 3, the properties of storage vesicles from SHR suggested that their responses to autonomic drugs might be different from those of NWR. Studies with reserpine have now been completed and will be published shortly (see publications list).

Reserpine administration resulted in a larger initial decline in adrenal catecholamines in spontaneously hypertensive rats (SHR) than in normotensive Wistar rats (NWR); the difference was eliminated by pretreatment with chlorisondamine. Reserpine also produced a larger increase in SHR catecholamines and dopamine β-hydroxylase several days later; chlorisondamine pretreatment did not prevent the increases, although it did slightly slow the increases. Vesicles from SHR or NWR incubated with reserpine in vitro demonstrated equivalent inhibition of $ATP\text{-}Mg^{2+}$-stimulated epinephrine uptake. Recovery of uptake was more rapid in SHR than in NWR after reserpine inhibition, and this was associated with a burst of new vesicle synthesis in the SHR; chlorisondamine pretreatment reduced the number of new, immature vesicles in reserpine-treated SHR. Both SHR and NWR secreted equal proportions of their adrenal catecholamine contents after nicotine administration. These data suggest that the sympatho-adrenal system of the SHR exhibits an enhanced reflex response to reserpine but that reserpine is equally effective in SHR and NWR in producing blockade of vesicular catecholamine transport; these alterations can affect markedly the actions of autonomic drugs in the SHR.

Similar studies are now underway with nicotine and chlorisondamine (a nicotinic blocking agent). In NWR, nicotine evokes a sympatho-adrenal discharge, resulting in catecholamine depletion, tyrosine hydroxylase induction, and formation of immature vesicles. Chlorisondamine blocks many of the effects of nicotine, but itself evokes some reflex activity. The effects of nicotine disappears upon chronic administration. We will evaluate in the near future whether the acute and chronic effects of nicotine and chlorisondamine are different in the SHR.

The above studies suggest a resemblance between the SHR and human labile hypertension - i.e., periods of low sympathetic activity coupled with hyper-responsiveness to reflex stresses. Preliminary results obtained with developing SHR indicate that, prior to 3 weeks of age (pre-

hypertensive phase) basal sympathetic activity is high (resembling human essential hypertension); these studies will be continued with other sources of support.

An offshoot of this project has been the development of drugs with reserpine-like sympatholytic activity. An additional publication (see list) details further progress made in this area. Two amine uptake mechanisms appeared to operate in isolated adrenal medullary storage vesicles; one site had a high affinity for epinephrine (Km=30 μM) and low capacity (Umax=20 nmols/100 μg of endogenous catecholamines), while the other had a low affinity (Km=2 mM) and a higher capacity (Umax=130 nmols). The low affinity site was non-specific and did not display competitive inhibition by agents which affected the high affinity, stimulated transport system. The high affinity system was inhibited in a purely competitive fashion by a variety of indoleamines and phenethylamines, but the two classes of compounds displayed different structure-activity relationships. Substitution on the α-carbon decreased the abilities of indoleamines to inhibit stimulated epinephrine uptake, but enhanced activity of phenethylamines. Ring hydroxylation reduced, and methoxylation eliminated, the inhibitory activity of tryptamine, but the same substituents markedly enhanced the activity of phenethylamines. Studies of compounds with restricted side-chain conformation indicated that a condensed structure favored activity in indoleamines while an extended chain enhanced inhibition by phenethylamines. Linear alkylamines of 5 or 6 carbon length were also able to inhibit active epinephrine uptake. None of the agents inhibited the non-stimulated uptake of component of metaraminol, which uses primarily the low affinity system. These data suggest that while indoleamines and phenethylamines do compete with epinephrine for attachment to the high affinity transport site in the vesicle membrane, the point of interaction is probably solely at the locus which binds the amine nitrogen; the remainder of the two types of molecule probably bind to at least two different sites adjacent to the N-binding area.

Publications from this project:

1. Maturation of the adrenal medulla. I. Uptake and storage of amines in isolated vesicles of the rat. T.A. Slotkin, Biochem. Pharmacol. 22:2023-2032 (1973).

2. Maturation of the adrenal medulla. II. Content and properties of catecholamine storage vesicles of the rat. T.A. Slotkin, Biochem. Pharmacol. 22:2033-2044 (1973).

3. Binding of amines to purified adrenal medullary storage vesicle membranes. T.A. Slotkin and N. Kirshner, Biochem. Pharmacol. 22: 2492-2497 (1973).

4. Hypothetical model of catecholamine uptake into adrenal medullary vesicles. T.A. Slotkin, Life Sci. 13:675-683 (1973).

5. Reserpine-like effects of harmine on isolated adrenal medullary storage vesicles. H.O. Green and T.A. Slotkin, Mol. Pharmacol. 9:748-755 (1973).

6. Secretion and recovery of catecholamines by the adrenal medulla. N. Kirshner and T.A. Slotkin, Biochem. Pharmacol., in press.

7. Drug-resistant effect of adenine nucleotides and magnesium on catecholamine efflux from isolated adrenal medullary storage vesicles. T.A. Slotkin and H.O. Green, Biochem. Pharmacol., in press.

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9. Adrenal medullary storage vesicles of the spontaneously hypertensive rat. T.A. Slotkin and H.O. Green, Biochem. Pharmacol., in press.

10. Structure-activity relationships for the reserpine-like actions of derivatives of beta-carboline. T.A. Slotkin, Life Sci., in press.

11. Effects of reserpine on the adrenal medulla of the spontaneously hypertensive rat. T.A. Slotkin, Brit. J. Pharmacol., in press.

12. Inhibition of epinephrine and metaraminol uptake into adrenal medullary vesicles by aralkyl- and alkylamines. T.A. Slotkin, T.R. Anderson, F.J. Seidler and C. Lau, Biochem. Pharmacol., in press.

13. Acute and chronic effects of nicotine on synthesis and storage of catecholamines in the rat adrenal medulla. T.A. Slotkin and F.J. Seidler, in preparation.

14. Sympatho-adrenal stimulation and inhibition by acute and chronic chlorisondamine administration to rats. T.A. Slotkin, S.M. Schanberg, F.J. Seidler, C. Lau, J. Bartolome, J. Cooke, Biochem. Pharmacol., submitted.

Abstracts:

1. Uptake and storage of amines in isolated adrenal medullary vesicles of developing rats. T.A. Slotkin, Fed. Proc. 32:783Abs. (1973).

2. Maturation of adrenal catecholamine storage vesicles of the rat. T.A. Slotkin, Pharmacologist 15:210 (1973).

3. Adrenal medullary vesicles of hypertensive rats. T.A. Slotkin and H.O. Green, Clin. Res. 22:13A (1974).

4. Effects of tryptamines on epinephrine uptake into adrenal medullary vesicles. T.A. Slotkin, F.J. Seidler and M.D. Abou-Donia, Pharmacologist 16:257 (1974).